

<110> MUNROE, Donald G. KAMBOJ, Rajender PETERS, Diana KOOSHESH, Fatemeh VYAS, Tejal B. GUPTA, Ashwani K.

<120> IDENTIFICATION OF LYSOLIPID RECEPTORS INVOLVED IN INFLAMMATORY RESPONSE

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<140> 09/222,995

<141> 1998-12-30

<150> 60/109,885

<151> 1998-11-25

<150> 60/080,610

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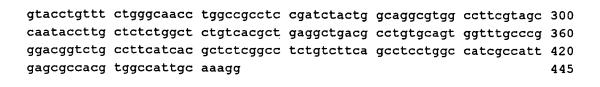
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185 190 195

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Arg Gln Val Ala Ser Ala Phe Ile Val Ile Leu Cys Cys Ala Ile Val 35 40 45

Val Glu Asn Leu Leu Val Leu Ile Ala Val Ala Arg Asn Ser Lys Phe 50 55 60

His Ser Ala Met Tyr Leu Phe Leu Gly Asn Leu Ala Ala Ser Asp Leu 65 70 75 80

Leu Ala Gly Val Ala Phe Val Ala Asn Thr Leu Leu Ser Gly Ser Val 85 90 95

Thr Leu Arg Leu Thr Pro Val Gln Trp Phe Ala Arg Glu Gly Ser Ala
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Phe Ile Thr Leu Ser Ala Ser Val Phe Ser Leu Leu Ala Ile Ala Ile 115 120 125

Glu Arg His Val Ala Ile Ala Lys Val Lys Leu Tyr Gly Ser Asp Lys 130 135 140

Ser Cys Arg Met Leu Leu Ile Gly Ala Ser Trp Leu Ile Ser Leu 145 150 155 160

Val Leu Gly Gly Leu Pro Ile Leu Gly Trp Asn Cys Leu Gly His Leu 165 170 175

Glu Ala Cys Ser Thr Val Leu Pro Leu Tyr Ala Lys His Tyr Val Leu 180 185 190

Cys Val Val Thr Ile Phe Ser Ile Ile Leu Leu Ala Ile Val Ala Leu 195 200 205

Tyr Val Arg Ile Tyr Cys Val Val Arg Ser Ser His Ala Asp Met Ala
210 215 220

Ala Pro Gln Thr Leu Ala Leu Leu Lys Thr Val Thr Ile Val Leu Gly 225 230 235 240

Val Phe Ile Val Cys Trp Leu Pro Ala Phe Ser Ile Leu Leu Leu Asp 245 250 255

Tyr Ala Cys Pro Val His Ser Cys Pro Ile Leu Tyr Lys Ala His Tyr 260 265 270

Xaa Phe Ala Val Ser Thr Leu Asn Ser Leu Leu Asn Pro Val Ile Tyr 275 280 285

Thr Trp Arg Ser Arg Asp Leu Arg Arg Glu Val Leu Arg Pro Leu Gln
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Cys Trp Arg Pro Gly Val Gly Val Gln Gly Arg Arg Gly Gly Thr 305 310 315 320

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Val Glu Asn Leu Leu Val Leu Ile Ala Val Ala Arg Asn Ser Lys Phe 50 55 60

His Ser Ala Met Tyr Leu Phe Leu Gly Asn Leu Ala Ala Ser Asp Leu 65 70 75 80

Leu Ala Gly Val Ala Phe Val Ala Asn Thr Leu Leu Ser Gly Pro Val
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Thr Leu Ser Leu Thr Pro Leu Gln Trp Phe Ala Arg Glu Gly Ser Ala 100 105 110

Phe Ile Thr Leu Ser Ala Ser Val Phe Ser Leu Leu Ala Ile Ala Ile
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Glu Arg Gln Val Ala Ile Ala Lys Val Lys Leu Tyr Gly Ser Asp Lys 130 135 140

Ser Cys Arg Met Leu Met Leu Ile Gly Ala Ser Trp Leu Ile Ser Leu 145 150 155 160

Ile Leu Gly Gly Leu Pro Ile Leu Gly Trp Asn Cys Leu Asp His Leu 165 170 175



Glu Ala Cys Ser Thr Val Leu Pro Leu Tyr Ala Lys His Tyr Val Leu 180 185 190

Cys Val Val Thr Ile Phe Ser Val Ile Leu Leu Ala Ile Val Ala Leu 195 200 205

Tyr Val Arg Ile Tyr Phe Val Val Arg Ser Ser His Ala Asp Val Ala

Gly Pro Gln Thr Leu Ala Leu Leu Lys Thr Val Thr Ile Val Leu Gly 225 230 235 240

Val Phe Ile Ile Cys Trp Leu Pro Ala Phe Ser Ile Leu Leu Leu Asp 245 250 255

Ser Thr Cys Pro Val Arg Ala Cys Pro Val Leu Tyr Lys Ala His Tyr 260 265 270

Phe Phe Ala Phe Ala Thr Leu Asn Ser Leu Leu Asn Pro Val Ile Tyr 275 280 285

Thr Trp Arg Ser Arg Asp Leu Arg Arg Glu Val Leu Arg Pro Leu Leu 290 295 300

Cys Trp Arg Gln Gly Lys Gly Ala Thr Gly Arg Arg Gly Gly Asn Pro 305 310 315 320

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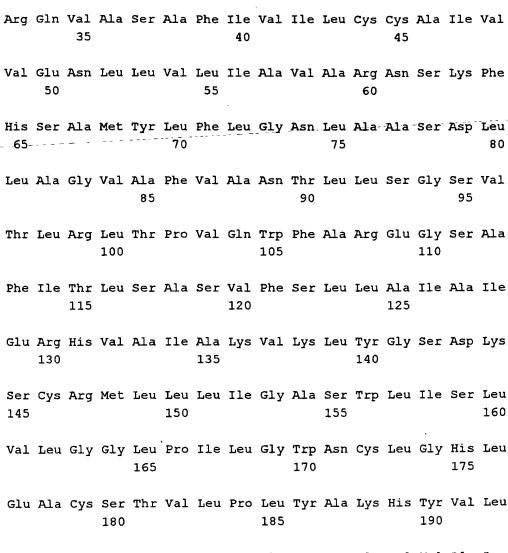
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Tyr Val Arg Ile Tyr Cys Val Val Arg Ser Ser His Ala Asp Met Ala 210 215 220

Ala Pro Gln Thr Leu Ala Leu Leu Lys Thr Val Thr Ile Val Leu Gly 225 230 235 240

Val Phe Ile Val Cys Trp Leu Pro Ala Phe Ser Ile Leu Leu Leu Asp 245 250 255

Tyr Ala Cys Pro Val His Ser Cys Pro Ile Leu Tyr Lys Ala His Tyr 260 265 270

Leu Phe Ala Val Ser Thr Leu Asn Ser Leu Leu Asn Pro Val Ile Tyr



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Cys Trp Arg Pro Gly Val Gly Val Gln Gly Arg Arg Gly Gly Thr 305 310 315 320

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Thr Asn Leu Leu Val Ile Ala Ala Ile Ala Ser Asn Arg Arg Phe His 50 55 60

Gln Pro Ile Tyr Tyr Leu Leu Gly Asn Leu Ala Ala Ala Asp Leu Phe 65 70 75 80

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Arg Cys Ser Arg Met Ala Pro Leu Leu Ser Arg Ser Tyr Leu Ala Val

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Lys Thr Val Val Ile Ile Leu Gly Ala Phe Val Val Cys Trp Thr Pro 245 250 255

Gly Gln Val Val Leu Leu Leu Asp Gly Leu Gly Cys Glu Ser Cys Asn 260 265 270

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Thr Phe Arg Arg Leu Leu Cys Cys Ala Cys Leu Arg Gln Ser Thr Arg 305 310 315 320

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<213> Homo sapiens

<400> 25

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13B). Therefore, induction of inflammatory gene expression pathways is a conserved feature of EDG-4 in humans and rats, and likely reflects a fundamental bidlogical aspect of receptor function.

Together, these results suggest that the SRE response is a shared feature of many different edg/lysolipid receptors, and can be used to verify the response of intact, functional receptors to their cognate agonist(s). On the other hand, the NF-kB response is shared by a subset of edg/lysolipid receptors which are specialized to mobilize inflammatory gene expression and immune system recruitment. Since EDG-1, EDG-3, EDG-4 and EDG-7 are all S1P/SPC receptors, their varying and even overlapping tissue distribution and inducibility frustrate the meaningful design, screening and therapeutic testing of anti-inflammatory S1P analogs unless the subtype specificity of inflammatory signaling is appreciated. This complexity highlights the value and utility of the recombinant inflammatory lysolipid receptors and the functional assays specified herein.

EXAMPLE 12. Identification of human expressed sequence tags (ESTs) homologous to rat H218 (EDG-4).

ISE® LOND: A BLAST search of the complete GenBank database was conducted with the sequence of an oligonucleotide RE4_181F [3'-GAGAAGGTTGAGGAACACTACAATTACACCAA GGA-3'], based on the sequence of rat EDG-4. The search identified a human EST (GenBank accession AA804628), which was 88% identical to the corresponding region of rat EDG-4 cDNA (GenBank accession U10699). A subsequent TBLASTN search of the EST database using the predicted polypeptide product of the rat EDG-4 cDNA (according to accession number U10699) revealed 2 other matching EST's (accession AA827835 and AA834537) in addition to the original human EST. The 3 EST's encompassed the predicted translation start site of human EDG-4 (based on similarity to rat EDG-4), overlapped each other extensively, and together spanned some 109 codons of the N-terminal portion of the human EDG-4 polypeptide (Figure 14). The predicted fragment of the human EDG-4 polypeptide showed 90.1% identity and 93.3% similarity to the equivalent fragment of rat EDG-4, suggesting the human polypeptide is an ortholog of the rat EDG-4 gene product, rather than a closely related gene product. A BLAST search was then conducted with the complete sequence of rat EDG-4 cDNA (accession number U10699) against the EST database. In addition to the previously identified EST's, 2 EST's apparently derived from the 3'-untranslated region of human EDG-4 cDNA adjacent to the

poly(A) tail were found (AA767046 and N93714). Of the 5 human EST's identified in total, only N93714 was present in the public database before February 19, 1998. This EST was derived from the 3' end of a 1421 bp cDNA insert which contained no coding region. The closest match recorded in the DBEST database entry (accession 500502) was a cGMP phosphodiesterase. The 5' end of the clone had been sequenced and given the GenBank accession W21101; however, similarity to other cDNAs was obscured by the presence of an Alu sequence.

EXAMPLE 13. Survey of potential cDNA sources using 5' end and 3' end diagnostic PCR.

- To evaluate possible sources of human EDG-4 cDNA from HeLa cells (which express the inflammatory S1P/SPC receptor) and lung (a predominant site of EDG-4 expression in rat) for the presence of the desired cDNA fragments, diagnostic PCR primers were designed from the cluster of 5' end EST's (AA804628, AA834537 and AA827835) and 3' end EST's (N93714 and AA767046): 5' end primers:
- 15 HE4-DF1 [5'-ATTATACCAAGGAGACGCTGGAAAC-3'] (SEQID NO:2)
 HE4-DR1 [5'-AGAGAGCAAGGTATTGGCTACGAAG-3'] (SEQID NO:3)
 3' end primers:

HE4-DF2 [5'-TCCTCTCCTCGTCACATTTCCC-3'] (SEQ 10 NO. 4)
HE4-DR2 [5'-GCATTCACAAGAAATTACTCTGAGGC-3'] (SEQ 10 NO.5)

Template sources: 1) cDNA library from WI-38 lung fibroblasts (Origene Technologies Inc., Cat. DLH-102); 2) cDNA library from human lung (Clontech, Cat. 7114-1); 3) cDNA library from HeLa cells (Invitrogen, Cat. A550-26); 4) First strand cDNA prepared in-house from HeLa cell total RNA. Each template was amplified with each pair of primers using the ExpandTM PCR system from Boehringer Mannheim (Cat.1681-842).

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Each reaction contained the following reagents:

	2 μ1	10x PCR Buffer 3
	0.4 μ1	25mM dNTP mix
30	0.6 μ1	Primer HE4-DF1 or HE4-DF2 (10 µM)
	0.6 µ1	Primer HE4-DR1 or HE4-DR2 (10 µM)

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Hold:

amplified, containing the complete coding region. These primers were used in a PCR reaction with the WI-38 human lung fibroblast cDNA library (Origene) as follows:

HE4-DF3 [5'-GAGCCCCACCATGGGCAGCTTGTACT-3'] (SEQ 1D NO.6)
HE4-DR2 [5'-GCATTCACAAGAAATTACTCTGAGGC-3'] (SEQ 1D NO.7)

Each reaction contained the following reagents:

	5 μι	10x PCR Burier 3
	1.0 μΙ	25mM dNTP mix
10	1.5 μ1	Primer HE4-DF3 (10 μM)
	1.5 μ1	Primer HE4-DR2 (10 μM)
	0.75 μ1	Expand TM enzyme (2 units)
	39.25 μ1	water
	1 μΙ	cDNA template (250 ng or 500 ng of DNA)

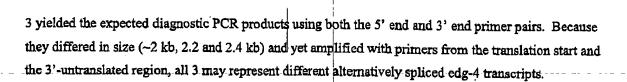
PCR conditions:

Incubate:	94°C for 2 min
10 cycles:	94°C for 40 sec
	60°C for 40 sec
	68°C for 5 min
25 cycles:	94°C for 40 sec
	60°C for 40 sec
	68°C for 3 min
Incubate:	68°C for 8 min

4°C

Amplified reactions from 250 ng (tube 227-45) and 500 ng (227-50) of cDNA template each contained 3

PCR products 2 kb or larger. The PCR reaction and the DNA fragments from the gel were purified using QIAquick PCR purification kit (Qiagen Cat. 28106) and QIAquick gel extraction kit (Qiagen, Cat. 28704), respectively. Diagnostic PCR reactions were carried out on each of the 3 PCR products, and all



The 3 PCR products were used as templates to reamplify human edg-4 with primers containing restriction sites suitable for cloning into an expression vector. Two different 3'-end primers were selected with longer (HE4-DR3) or shorter (HE4-DR4) 3'-untranslated regions. The following PCR primers and PCR conditions were used:

10	HE4-DF4	[5'-TTTAAAAAGCTTCCCACCATGGGCAGCTTGTACT-3'] (SEQ ID NO: 8)
	HE4-DR3	[5'-TATATATCTAGAGAAATGTGACGAGGAGAGG-3'] (SEQ ID NO: 9) [5'-TATATATCTAGAGGAAATGTGACGAGGAGAGG-3'] (SEQ ID NO: 10)
	HE4-DR4	[5'-TATATATCTAGAGGAAATGTGACGAGGAGAGG-3'] (SEO ID NO: 10)

Each reaction contained the following reagents:

15	5 μ1	10x PCR Buffer 3
	1.0 μ1	25mM dNTP mix
	1.5 μ1	Primer HE4-DF4 (10 μM)
	1.5 μ1	Primer HE4-DR3 or HE4-DR4 (10 μM)
	0.75 μ1	Expand TM enzyme (5 units)
20	39.25 µl	water
	1 μ1	DNA

PCR conditions:

Incubate: 94°C for 2 min
25 28 cycles: 94°C for 40 sec

60°C for 40 sec

68°C for 3.5 min

Incubate: 68°C for 8 min

30 Hold: 4°C



A pair of primers was designed from two ends of reading frame of human edg-4 cDNA sequence to engineer the edg-4 open reading frame into a vector designed for GFP fusion protein expression, with the GFP tag carboxy-terminal to the full-length EDG-4 polypeptide:

5 5'-End Primer: Contains Site for Kpn I enzymel and optimized (Kozak) translation initiation sequence:

HE4-ATG KpnF: [5'-TTTAAAGGTACCGCCACCATGGGCAGCTTGTAC-3'] (SEQ ID NO:11')

3'-End Primer: Contains site for XbaI enzyme, and lacks naturally-occurring edg-4 stop codon:

HE4-xbs/1096R: [5'-TATATATCTAGAGACCACCGTGTTGCCCTCCAG-3'] (SEQ ID NO. 12)

pc3-hedg4#36 plasmid DNA was amplified with the above pair of primers under the following conditions of PCR amplification, using the ExpandTM PCR system from Boehringer Mannheim (Cat.

15 1681-842).

The reaction contained the following reagents:

5 μl of 10x PCR Buffer 3

20 1.0 μ l of 25mM dNTP mix

1.5 µl of Primer HE4-ATG KpnF (10 pmol/l)

1.5 µl of Primer HE4-xba/1096R (10pmol/l)

 $0.75 \mu l$ of Enzyme (2 units)

39.25 µl water

25 1 µl DNA

PCR conditions:

Incubate:

94°C for 2 min

10 cycles:

94°C for 1 min

30

50°C for 1 min

62

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